2.0 METHODS FOR *IN VITRO* AR TA ASSAYS

2.1 Introduction

There are no standardized methods for performing AR TA assays. The majority of published AR TA studies were conducted to investigate the process of AR-induced transcriptional activation or to identify structure-activity relationships; relatively few studies were designed to assess the ability of a test substance to act as an AR agonist or antagonist. Furthermore, very few studies have been conducted using the same cell line, AR construct, and reporter gene construct (**Table 2-1**). The 27 reports reviewed in this BRD (26 peer-reviewed publications and one submitted report containing unpublished data) described studies using yeast (*Saccharomyces cerevisiae*), nine different mammalian cell lines, and one fish cell line (EPC). The mammalian cell lines used included six human (HepG2, HeLa, LnCaP-FGC, MDA-MB-453 and its derivative MDA-MB-453-kb2, PC-3 and its derivative PALM), two monkey (CV-1, COS-1), and one using Chinese hamster ovary (CHO) cells. The majority of published AR TA studies used cells that were transiently transfected with the AR. Thus, new transiently transfected cells were produced for each experiment, and the sensitivity and/or responsiveness of each batch of transfected cells were determined by the characteristics of the cell line and the constructs used, and by the efficiency of transfection. Less frequently used were cells stably transfected with a plasmid containing the gene coding for the AR (HeLa, PALM, CHO, yeast), or those containing an endogenous AR (LnCaP-FGC, MDA-MB-453). The human AR (hAR) was used in all but two of the studies included in this BRD; these two studies used cells transfected with trout and mouse AR.

All but one of the AR TA assays considered in this BRD used a reporter gene to assess TA. The non-reporter gene based test method used the induction of cell proliferation as an indicator of transcriptional activation (Sonnenschein et al., 1989). In assays that use transiently transfected cells, the cells are transfected with an expression plasmid and/or a reporter plasmid. The plasmid known as the expression construct contains the AR that is under the control of a viral promoter gene (often from SV-40).

Table 2-1 Cell Lines, Plasmids, and Reference Androgens Used in *In Vitro* AR TA Assays

Cell Line	Species	Tissue or Strain	AR*	AR Plasmid[a]	Reporter Plasmid[a]	Other Plasmids	Reporter gene	# Sub. Tested	Ref. Agonist	Ref. Antag.	Reference
CHO	Chinese hamster	Ovary	h	hAR(stable)	MMTV-*Luc* (stable)		luciferase	9	DHT		Deckers et al. (2000)
CHO	Chinese hamster	Ovary	h	pZeoSV2AR (stable)	pIND ARE B10-*Luc* (stable)		luciferase	12	DHT	DHT	Otsuka Pharmaceutical Co. (2001)
CHO	Chinese hamster	Ovary	h	pCMV3.1.hAR (transient)	MMTV-CAT (transient)	SV40-pCH110 (β-gal)	CAT	9	T		Deslypere et al. (1992)
CHO	Chinese hamster	Ovary	h	pSVAR0 (transient)	MMTV- *Luc* (transient)		luciferase	11	R1881	R1881	Vinggaard et al. (1999)
CHO	Chinese hamster	Ovary	h	pSVAR0 (transient)	MMTV-*Luc* (transient)		luciferase	10	R1881	R1881	Vinggaard et al. (2000)
CHO	Chinese hamster	Ovary	h	pSVAR0 (transient)	MMTV-*Luc* (transient)		luciferase	4	R1881	R1881	Bonefeld-Jorgenson et al. (2001)
CHO	Chinese hamster	Ovary	h	pZeoSV2AR (transient)	pIND ARE B10-*Luc* (transient)	p-EGFP	luciferase	65		DHT	Otsuka Pharmaceutical Co. (2001)
CV-1	Monkey	Kidney	h	Ad5 hAR (transduced)	MMTV-*Luc* (transduced)		luciferase	9	DHT	DHT	Hartig et al. (2002)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV-*Luc* (transient)		luciferase	9		DHT	Kelce et al. (1995)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV-*Luc* (transient)		luciferase	5	DHT	DHT	Kemppainen and Wilson (1996)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	pMTV29VTM (transient)		CAT	9	R1881	R1881	Kemppainen et al. (1992)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV-*Luc* (transient)		luciferase	12	DHT	DHT	Kemppainen et al. (1999)
CV-1	Monkey	Kidney	h	pCMVhAR (transient)	MMTV-*Luc* (transient)		luciferase	2		DHT	Lambright et al. (2000)

Cell Line	Species	Tissue or Strain	AR*	AR Plasmid[a]	Reporter Plasmid[a]	Other Plasmids	Reporter gene	# Sub. Tested	Ref. Agonist	Ref. Antag.	Reference
CV-1	Monkey	Kidney	mo	Not provided (transient)	pSV2-CAT (transient)		CAT	3	DHT		Van Dort et al. (2000)
EPC	Carp	Skin tumor	tr	pCMV-rtAR-α(transient)	pARE3TK-CAT (transient)		CAT	8			Takeo and Yamashita (2000)
HeLa (E19)	Human	Cervical cancer	h	pTetCMV-F0(S)-AR (stable)	MMTV-*Luc* (transient)		luciferase	4	T		Wang and Fondell (2001)
HeLa (E19)	Human	Cervical cancer	h	pTetCMV-F0(S)-AR (stable)	ARE2-DS-*Luc* (transient)		luciferase	4	T		Wang and Fondell (2001)
HeLa (E19)	Human	Cervical cancer	h	pTetCMV-F0(S)-AR (stable)	PB(-285/+32) *Luc* (transient)		luciferase	1			Wang and Fondell (2001)
HepG2	Human	Hepatoma	h	pRSAR (transient)	MMTV-*Luc* (transient)	pCMV β-gal	luciferase	15		DHT	Gaido et al. (2000)
HepG2	Human	Hepatoma	h	pRSAR (transient)	MMTV-*Luc* (transient)	pCMV β-gal	luciferase	14	DHT	DHT	Maness et al. (1998)
HepG2	Human	Hepatoma	h	pRSAR (transient)	MMTV-*Luc* (transient)	pCMV β-gal	luciferase	2	DHT	DHT	Tamura et al. (2001)
LnCaP-FGC	Human	Metasticized prostate adeno-carcinoma	h	Endogenous AR	NA**		Cell growth	16	T		Sonnenschein et al. (1989)
MDA-MB-453	Human	Breast carcinoma	h	Endogenous AR	Ad/m*Luc*7 (transduced)		luciferase	8	DHT	DHT	Hartig et al. (2002)
MDA-MB-453-kb2	Human	Breast carcinoma	h	Endogenous AR	MMTV-*Luc* (stable)		luciferase	2		DHT	Lambright et al. (2000)
MDA-MB-453-kb2	Human	Breast carcinoma	h	Endogenous AR	MMTV-*neo*-*Luc* (stable)		luciferase	13		DHT	Wilson et al. (2002)

Cell Line	Species	Tissue or Strain	AR*	AR Plasmid[a]	Reporter Plasmid[a]	Other Plasmids	Reporter gene	# Sub. Tested	Ref. Agonist	Ref. Antag.	Reference
PALM (PC-3)	Human	Prostate adeno-carcinoma	h	PSG_5-puro-hAR (stable)	MMTV-*neo*-*Luc* (stable)		luciferase	12		R1881	Sultan et al. (2001)
PALM (PC-3)	Human	Prostate adeno-carcinoma	h	PSG_5-puro-hAR (stable)	pMMTV-*neo*-*Luc* (stable)		luciferase	17	M	M	Terouanne et al. (2000)
PALM (PC-3)	Human	Prostate adeno-carcinoma	h	pCMV5-hAR (stable)	MMTV. pMAM.neo.Luc (stable)		luciferase	21	DHT	DHT	Schrader and Cooke (2000)
PC-3	Human	Prostate adeno-carcinoma	h	PSG_5-puro-hAR (transient)	MAM-*neo*-*Luc* (transient)	pCMV-β-gal	luciferase	4			Terouanne et al. (2000)
Yeast	*S. cerevisiae*	Not provided	h	Not provided (stable)	LacZ (stable)		β-gal	8	DHT	DHT	Moffat et al. (2001)
Yeast	*S. cerevisiae*	YPH500	h	CUP1-Met hAR (stable)	Not provided (stable)		β-gal	7	DHT	DHT	O'Connor et al. (1998)
Yeast	*S. cerevisiae*	YPH500	h	CUP1-Met hAR (stable)	Not provided (stable)		β-gal	4	DHT	DHT	O'Connor et al. (1999)
Yeast	*S. cerevisiae*	YPH500	h	CUP1-Met hAR (stable)	Not provided (stable)		β-gal	5	DHT	DHT	O'Connor et al. (2000)
Yeast	*S. cerevisiae*	YPH500	h	CUP1-Met hAR (stable)	YRPGALE1 (stable)		β-gal	22	DHT		Gaido et al. (1997)

Abbreviations: Antag. = Antagonist, DHT = 5α-Dihydrotestosterone, β-gal = beta-Galactosidase, M = Mibolerone, Ref. = Reference, R1881 = Methyltrienolone, Sub. = Substances, T = Testosterone.

[a]Stable, transient, and transduced in parenthesis indicate whether the plasmid was stably or transiently integrated, or transduced with adenovirus, respectively, into the cell.

*AR refers to the source of the androgen receptor; h = human, mo = mouse, and tr = trout.

**NA refers to the fact that cell proliferation was measured in this assay, and thus no reporter construct was required.

*** Not all publications included information on the precise composition of the various vectors.

The reporter plasmid contains the hormone responsive elements (HRE) controlling the expression of a reporter gene, usually luciferase (*Luc*), chloramphenicol acetyltransferase (*CAT*) or, in yeast, β-galactosidase (β-*gal*). Since the HRE sequence is contained within the mouse mammary tumor virus long terminal repeat, this DNA sequence is frequently used as the source of the HRE. In AR TA assays that use stably transfected cells, the cells can contain the stably integrated expression plasmid only or both the expression and the reporter plasmids. In the former case, such cells are transiently transfected with the reporter plasmid. Using either transient or stably transfected cells, when the transfected cells are exposed to a substance that interacts with the AR, the AR becomes activated by a change in its conformation. The activated AR binds with soluble cell factors, and then the complex binds to the AR response elements on the second plasmid. This binding initiates the expression of the reporter gene and the production of its associated enzyme. An appropriate substrate added to the incubation mixture is metabolized by the enzyme resulting in the production of an easily detected product. The majority of AR TA studies use luciferase to assess transcriptional activation because its use makes the assay more rapid, more sensitive, and easier to perform than CAT-based assays. Also, in contrast to luciferase-based assays, CAT-based assays require a radiolabeled substrate (either chloramphenicol or acetyl-CoA).

Cytotoxicity can be a complicating factor in AR TA assays, particularly when antagonism is being assessed. The absence of or a decrease in the AR transcriptional activation response might be the result of cell toxicity rather than reflecting the ability of the test substance to interact with the AR. Cell toxicity can be corrected for by performing a parallel cytotoxicity experiment or by measuring the product of a constituitively active gene transfected into the cell on a separate plasmid. Some of the mammalian cell lines transfected with *Luc* or *CAT* reporter constructs have also been transfected with a plasmid coding for the β-*gal* gene. The synthesis of β-galactosidase is independent of a receptor-mediated effect, and a comparison of its level in treated versus control cells can be used as a measure of treatment-related cell toxicity.

In studies to measure agonism, the cells are treated with a test substance and the induction of luciferase, CAT, or β-galactosidase measured. To assess relative potency, the response obtained with the test substance can be compared with the response obtained when the cells are treated

with a reference androgen (e.g., DHT, R1881). In studies to determine antagonism, the cells are treated simultaneously with the test substance and the reference androgen and the ability of the test substance to inhibit transcriptional activation is measured.

Because there are no "consensus" cell lines, vectors, or specific treatment protocols for AR TA studies, the following sections describe general protocols for agonism and antagonism studies using mammalian or yeast cells transfected with a reporter gene, and mammalian cells using growth as an endpoint.

2.2 Mammalian Cell AR TA Reporter Gene Assays

2.2.1 Expression and Reporter Gene Constructs

For transfection into mammalian cells, the recombinant plasmid is constructed by ligating the cDNA of the AR gene into a eukaryotic expression vector that contains the viral early gene promoter SV-40, the human growth hormone transcription termination and polyadenylation signals, the SV-40 origin of replication, and an antibiotic resistance gene for selection. An alternative to the SV-40 gene used in some studies is the CMV early gene promoter. Also, a number of genes with different termination and polyadenylation signals have been used in the various expression constructs used in AR TA studies.

The *Luc* reporter plasmid contains the *Luc* gene regulated by the glucocorticoid-inducible HRE found in the mammary mouse tumor virus long terminal repeat. The *CAT* reporter plasmid pMTV29VTM contains two glucocorticoid response elements separated by 29 base pairs and positioned 5' to the *CAT* gene. It is important to note that the MMTV promoter sometimes used in the reporter plasmid, can be regulated by the GR and progesterone receptor (PR) and that certain compounds may interact with the AR and also with either the PR or GR. Unless the investigator is cognizant of this possible cross reactivity, the data obtained with certain substances may not truly reflect AR-induced transcriptional activation. These potential interferences can be compensated for by adding a specific chemical that blocks the activation of the GR or PR.

2.2.2 Stably and Transiently Transfected Cell Lines

The majority of AR TA studies considered for this BRD used transiently transfected cells, despite the fact that a new batch of transfected cells must be produced for each new experiment. Transfection is performed by exposing the cells to both plasmids in the presence of, for example, calcium phosphate, DEAE dextran, or a lipofection agent such as FuGene™. These substances increase cell membrane permeability, allowing for the passive uptake of the plasmids by the cells. These foreign DNAs are typically rejected by the cell within three to seven days after transfection. In cells that harbor an endogenous or stably transfected AR, only the reporter construct and perhaps the construct to assess cytotoxicity is transfected. The transfected cell lines that have both constructs either stably incorporated into their genome or as stable plasmids in the cell are easier to use since they do not require genetic manipulation before performing the assay.

2.2.3 *In Vitro* Mammalian Cell AR TA Assays with a Reporter Gene

Mammalian cells at the recommended density for the particular cell line are seeded into culture dishes or wells of microtiter plates and cultured for 18 to 24 hours at 37°C. The cells are transfected with the appropriate plasmids using either calcium phosphate, DEAE dextran, or a lipofection agent. After attachment for 4-24 hours at 37°C to express the AR, the cells are treated with the test substance dissolved in the culture medium or other appropriate solvent, such as absolute ethanol or dimethyl sulfoxide (DMSO). The cells are incubated for 24 to 48 hours at 37°C. The medium is aspirated, the cells are washed with an appropriate buffer and then lysed with the same buffer containing $MgCl_2$, Triton X 100, and dithiothreitol, or other agents appropriate to the reporter construct used. After 15 minutes at room temperature and centrifugation, if necessary, for a short time to sediment cell debris, an aliquot of the supernatant is removed to measure induction of the reporter gene product. For the induction of luciferase, adenosine triphosphate (ATP) and coenzyme A are added in glycylglycine buffer to the cell lysate in a microtiter plate. Luciferin is added to start the reaction and the luminescence is measured using a microtiter plate luminometer. The data are expressed in relative light units. For the induction of CAT, an aliquot of the lysed cells is incubated with radiolabeled chloramphenicol and acetyl coenzyme A (Gorman et al., 1982). The extracts are incubated for 30 minutes at 37°C with samples removed at various time points. The reaction is stopped with

ethyl acetate, which is used to extract the acetylated chloramphenicol. The organic phase is dried, redissolved in ethyl acetate, and spotted on silica gel plates. The radioactive acetylated product is separated from the parent chloramphenicol using thin layer chromatography. The radioactive spots are located following autoradiography of the plates for 18 hours, cut out, and counted in a scintillation counter. When β-galactosidase is used as a measure of toxicity, the enzyme activity is measured using *o*-nitrophenyl-β-galactoside (ONPG) as the substrate. Following hydrolysis of OPNG by β-galactosidase, the intensity of the yellow product is measured using a spectrophotometer.

In agonism studies, the cells are treated with a test substance and the induction of the reporter gene and its associated product are used to indicate a positive response. To assess relative potency, the maximal fold-increase induced by the test substance can be compared with that induced by the reference androgen or, where dose-response data are generated, EC_{50} for the test substance and the reference androgen can each be calculated and compared. A reference androgen (e.g., DHT, R1881) is included not only for an assessment of relative potency but also to demonstrate the adequacy of the test system. For antagonism studies, the cells are exposed simultaneously to the reference androgen and the test substance while control cells are exposed to the reference androgen only. The difference in induction of the reporter gene product in the presence and absence of the test substance is used as a measure of antagonism.

2.3 Yeast Cell AR TA Reporter Gene Assays

2.3.1 Expression and Reporter Gene Constructs

The yeast expression plasmid contains the CUP metallothionein promoter fused to the cDNA of hAR. The reporter plasmid carries two copies of the androgen response element upstream of β-*gal*.

2.3.2 Yeast Cell AR TA Assays with a Reporter Gene

In the yeast assay, various strains of *S. cerevisiae* with a stably transfected hAR and a construct containing the β-*gal* reporter gene are grown overnight at 30°C in an orbital shaker in a selective medium containing a yeast nitrogen base and ammonium sulfate. The next day, an aliquot of the overnight culture is grown to mid-log phase. This suspension is diluted and the test substance

dissolved in medium, ethanol, or DMSO is added. As the hAR in these cells is linked to a copper metallothionein promoter, copper sulfate ($CuSO_4$) is added to the yeast to induce receptor production. The cells are incubated overnight at 30°C with vigorous shaking and the optical density (OD) is read at 600 nm to assess cell growth or toxicity. A diluted aliquot of the cells is pipetted into a microtiter plate. Assay buffer containing OPNG and a lysing solution containing sodium dodecyl sulfate (SDS), mercaptoethanol, and oxalyticase is added to the cells. The increase in production of *o*-nitrophenol by the induced β-galactosidase is measured at 420 nm using a microtiter plate reader. The OD is also measured at 550 nm to correct for colorimetric distortion due to debris. β-Galactosidase activity is calculated according to the Miller equation,

$$\text{Miller Units } (A_{420}/\text{min/mL cells/OD}_{600}) = 1000 \times \frac{OD_{420} - (1.75 \times OD_{550})}{T \times V \times OD_{600}}$$

where T = minutes of reaction time and V = volume of assay in mL.

In agonism studies, the yeast cells are treated with the test substances and the induction of β-galactosidase is measured. A positive response is indicated by a dose-related increase in the induction of β-galactosidase. For an assessment of relative potency, the induction may be compared to the results from a reference androgen. For antagonism studies, the cells are exposed simultaneously to the reference androgen and the test substance; control cells are exposed to the reference androgen only. The difference in β-galactosidase activity in the presence and absence of the test substance is used as a measure of androgen antagonism.

2.4 *In Vitro* Mammalian Cell AR TA Assays using Growth as an Endpoint

Mammalian cells (LnCaP) containing an endogenous hAR are seeded in 12-well plates in the presence of 5% fetal bovine serum and grown for 48 to 72 hours at 37°C to allow the cells to attach to the plastic surface (Sonnenschein et al., 1989). The medium is removed and replaced with fresh medium containing human serum that has been charcoal stripped to remove contaminating hormones. Various concentrations of the test substance are added and the cells are grown for seven days at 37°C. A number of procedures can be used to quantitate total cell

growth. For example, cell lysing solution is added to the wells and the cell nuclei are counted using a Coulter counter. The parameter of growth generally considered is relative proliferative potency (RPP). This parameter is calculated as the ratio (x100) between the concentration of the reference androgen and the test substance that was required to elicit a maximal cell yield after seeding 10,000 cells/well.

2.5 Reference Androgens

The majority (21 of 31, 68%) of AR TA studies considered in this BRD used DHT as the reference androgen for agonist/antagonist studies. R1881 was used as the reference androgen in five AR TA studies (16%), testosterone in four studies (13%), and mibolerone in one study (3%).